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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/903,396	07/10/2001	Keith D. Allen	R-359	9463

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DeltaGen, Inc.
740 Bay Road
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EXAMINER	
BERTOGLIO, VALARIE E	

ART UNIT	PAPER NUMBER
1632	

DATE MAILED: 11/05/2003

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	09/903,396	ALLEN, KEITH D.	
	Examiner	Art Unit	
	Valarie Bertoglio	1632	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *15 August 2003* .

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-4,13-16,30-32 and 34-48* is/are pending in the application.

4a) Of the above claim(s) *1-4,13-16,30-32,and 34-35* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *36-48* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

11)☒ The proposed drawing correction filed on *15 August 2003* is: a)☒ approved b)☐ disapproved by the Examiner.

If approved, corrected drawings are required in reply to this Office action.

12)☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. §§ 119 and 120

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____ .

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

14)☒ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).

a) ☐ The translation of the foreign language provisional application has been received.

15)☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449) Paper No(s) _____ .
4) ☐ Interview Summary (PTO-413) Paper No(s). _____ .
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: .

DETAILED ACTION

Applicant's amendment filed on 08/15/2003 has been entered. Claims 5-12,17-19 and 33 have been canceled. Claims 36-48 have been added. Claims 1-4,13-16,30-32,34-35 and 36-48 are pending and claims 36-48 are under consideration in the instant action.

Election/Restrictions

Claims 1-4,13-16,30-32 and 34-35 are withdrawn from further consideration pursuant to 37 CFR 1.142(b), as being drawn to a nonelected invention, there being no allowable generic or linking claim. Applicant timely traversed the restriction (election) requirement in Paper No. 10.

Drawings

The drawings were received on 08/15/2003. These drawings are acceptable.

Sequence Compliance

The instant application is now sequence compliant.

Claim Rejections - 35 USC § 101/112

35 U.S.C. 101 reads as follows:

> Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Newly added claims 36-48 are rejected under 35 U.S.C. 101 because the claimed invention is not supported by either a specific and substantial asserted utility or a well-established utility.

The claims are directed to a transgenic mouse whose genome comprises a homozygous disruption in glucocorticoid-induced receptor gene, wherein the mouse exhibits hyperactivity, reduced anxiety, decreased propensity toward behavioral despair or decreased propensity toward depression (claims 36-40 and 48) and methods of making said mouse (claim 47). Claims are further directed to cells or tissues isolated from the same mouse (claim 41). Claims are also directed a mouse comprising a heterozygous disruption in glucocorticoid-induced receptor gene (claims 42-46).

The instant specification has contemplated that the nucleotide sequence set forth in SEQ ID NO: 1 encodes a glucocorticoid-induced receptor. The instant specification has further contemplated that disruption of the nucleotide sequence set forth in SEQ ID NO: 1 in a mouse will produce a phenotype associated with a disruption of a glucocorticoid-induced receptor. The instant specification has purported that such mice may be used to identify agents that modulate or ameliorate a phenotype associated with a disruption in SEQ ID NO: 1. See page 19, line 24-page 20, line 2.

The specification has provided general assertions that the claimed transgenic mice may be used to identify agents that affect a phenotype related to the mice. As such, the asserted utility, for the transgenic mouse encompassed by the claims, of screening agents that may affect

a phenotype of said mouse as provided by the instant specification and encompassed by the claims, does not appear to be specific and substantial. The asserted utility does not appear specific and substantial to the skilled artisan since the evidence of record has not provided any suggestion of a correlation between a homozygous disruption of a glucocorticoid-induced receptor gene, reduced anxiety, decreased propensity toward behavioral despair or decreased propensity toward depression, and any disease or disorder. Since the evidence of record has not provided a correlation between reduced anxiety, decreased propensity toward behavioral despair or decreased propensity toward depression and any disease or disorder, the utility of identifying agents that affect reduced anxiety, decreased propensity, toward behavioral despair or decreased propensity toward depression is not apparent. The evidence of record has not provided any other utilities for the transgenic mouse encompassed by the claims that are specific and substantial.

The instant specification has disclosed a transgenic mouse whose genome comprises a disruption in SEQ ID NO: 1, wherein the mouse exhibits hyperactivity, reduced anxiety, decreased propensity, toward behavioral despair or decreased propensity toward depression. See pages 53-54. The instant specification has discussed that the animals and cells of the instant invention can be used as models of disease (refer to pages 18-19). Specifically, the specification states that agents can be identified on the basis of their ability to affect at least one phenotype associated with a disruption in a glucocorticoid-induced receptor gene (page 19, lines 24-26). However, the evidence of record, while contemplating that the phenotypes inhibited by the claimed transgenic mice are associated with a disease, does not provide a correlation between the phenotypes of the claimed mouse and any disease or disorder, leaving the skilled artisan to speculate and investigate the uses of the transgenic mouse encompassed by the claims. While

the art at the time of filing taught using mice displaying phenotypes of increased anxiety or depression for screening agents for therapeutic activity (Gass, 2001, Physiology and Behavior, Vol. 73, pp. 8111-825, specifically, page 815-816, section 5 and page 820-821m section 8). The utility of these mice, however, does not reflect a use for the claimed mice displaying an opposite phenotype indicating decreased propensity toward depression, decreased propensity for behavioral despair or reduced anxiety. Mice with decreased propensity for anxiety or depression would not offer the same utility in screening for therapeutic agents to treat diseases such as anxiety or depression and the specification fails to correlate decreased propensity for anxiety or depression with any other disease. Furthermore, as taught by Gass et al., the usefulness of mutant mice as models of depression is not even clear without assessing that they specifically reflect human depression. The specification essentially gives an invitation to experiment wherein the artisan is invited to elaborate a functional use for the transgenic mouse encompassed by the claims. In light of the above, the skilled artisan would not find the asserted utility of the transgenic mouse encompassed by the claims to be specific and substantial.

Claims 6,8-10,23, 29-32 and 35-39 are also rejected under 35 U.S.C. 112, first paragraph. Specifically, since the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility for the reasons set forth above, one skilled in the art clearly would not know how to use the claimed invention.

Upon overcoming the utility and enablement rejections set forth above, the following issues of enablement under 35 USC 112-1st paragraph must also be addressed.

1) The specification fails to enable disrupting any glucocorticoid-induced receptor gene in a mouse or any other species or a cell other than a mouse cell. The claims lack a modifier before the phrase "endogenous mouse glucocorticoid-induced receptor gene" and therefore the breadth of the claims includes mouse glucocorticoid-induced receptor genes other than that set forth by SEQ ID NO:1. The evidence of record teaches only one glucocorticoid-induced receptor gene (Harrigan, 1991, Molecular Endocrinology, Vol. 5, pages 1331-1338; and the instant specification, SEQ ID NO:1). The specification does not provide adequate guidance for determining any other glucocorticoid-induced receptor gene or that other glucocorticoid-induced receptor genes have the same function as the glucocorticoid-induced receptor gene disclosed. Inserting the word "the" prior to the phrase "endogenous mouse glucocorticoid-induced receptor gene", would overcome this rejection.

2) The rejection based on the specification failing to enable making or using any transgenic mouse comprising a disruption in the glucocorticoid-induced receptor gene wherein the mouse is of any genetic background and wherein the mice exhibit hyperactivity, reduced anxiety, decreased propensity towards behavioral despair, or decreased propensity toward depression is maintained for reasons of record set forth on pages 12-14 of the previous office action.

Applicant's arguments with respect to this aspect of the rejection have been fully considered and are not considered persuasive. Applicant argues that one of skill in the art would be able to easily determine the phenotype of the claimed mice regardless of genetic background. However, the claims encompass generating the claimed mice with specific behavioral phenotypes using mice of any genetic background. As discussed in the previous office action

(refer to paragraph bridging pages 12-13), the state of the art was that genetic background has a significant effect on the development of the claimed phenotypes in knockout mice (refer to Crabbe and Yoshikawa). Furthermore, Liu taught that the response on the tail suspension test varies among different strains of mice (2001, Biological Psychiatry, Vol. 49, pages 575-581, specifically, page 576, column 1, lines 17-19; page 577, column 2, Results paragraph 1). More specifically, Mayorga taught that the occurrence of tail climbing in C57BL/6 mice in response to the tail suspension test, which is not observed in other strains, is an important consideration and limitation when using this strain in the tail suspension test (2001, Psychopharmacology, Vol. 155, pages 110-112, specifically page 110, paragraph bridging columns 1 and 2; page 111, column 2, paragraph 2). Mayorga also teaches that this phenomenon should be considered when planning experiments to characterize potential antidepressants in mice using the tail suspension test and in the choice of mouse strain for the generation and testing of knockout mice (page 111, column 2, last 5 lines). The reports of Crabbe, Yoshikawa, Liu and Mayorga are each evidenced in the specification as N0 generation animals did not display results in the open field test or the tail suspension test indicating they may be more hyperactive and less anxious or that they may have less of a propensity towards behavioral despair or depression than their wild-type littermates; however, N1 generation mice did display such results (page 53, last paragraph; Table 1; page 54, lines 4-9). The difference in the N0 and N1 generation animals is only the genetic background; N1 mice were backcrossed to the C57BL/6 strain whereas the N0 mice were not (page 53, lines 16-21). Furthermore, the specification states that "The discrepancy in the results observed in the Open Field and Tail Suspension Tests between generations may reflect differences in the background strains used to generate the mice" (page 54, lines 13-15). Thus, the

specification teaches that genetic background alters the phenotype of mice comprising a disruption in the glucocorticoid-induced receptor gene and fails to teach how to generate the claimed phenotypes in the mice of any genetic background as broadly encompassed by the claims.

3) The specification fails to enable the method of claim 47. The term "murine" in steps (a) and (b) encompass both mouse and rat species. The method is drawn to generating a mouse. The specification does not teach generating a mouse using an ES cell derived from any species other than mouse. Furthermore, as stated in the previous office action, the art at the time of filing was that totipotent ES cells that contribute to the germline had not been identified for any species other than mouse (refer to Mullins; Campbell and Wilmut). Therefore, ES cells derived from non-mouse species cannot be used to generate a transgenic animal. Claim 47 should be limited to producing a transgenic mouse using mouse embryonic stem cells.

4) The breadth of claims 42-48 is such that they encompass chimeric animals (genetic mosaics) wherein only a portion of the cells of the animal comprises the claimed genetic disruption. The specification teaches making transgenic animals whose genome comprises a homozygous disruption in the glucocorticoid-induced receptor gene in all somatic and germ cells wherein the mice display hyperactivity, reduced anxiety, decreased propensity towards behavioral despair, or decreased propensity toward depression. The specification does not teach a chimeric animal with these phenotypes. The method of making genetic mosaic animals is such that each resulting chimera is comprised of a different, unpredictable ratio of cells of various genotypes. This ratio cannot be predetermined. Furthermore, the spatial distribution of cells of each genotype cannot be predetermined. Therefore, the phenotype of chimeric animals is not

only dependent upon the genotype of the cells (which is unpredictable as set forth by the state of the art outlined on pages 8-10 of the previous office action; for example see Leonard; Moens;Griffiths, Mullins 1989,1990; Taurog) but is also dependent upon the spatial distribution of the cells and their relative population size. Thus, the phenotype of the chimeric animals encompassed by the claims is highly unpredictable. It would require undue experimentation for one of skill in the art to determine how to overcome the unpredictability associated with making chimeric animals such that the proportion and population of cells harboring a genetic alteration could be controlled in such a way as to increase the predictability of the phenotype of the resulting chimeric animal. Replacing the term "comprising" in line 1 of claims 42 and 47 with the phrase "whose genome comprises" is suggested.

5) Claims 42-48 encompass transgenic mice comprising a heterozygous disruption in the endogenous mouse glucocorticoid-induced receptor gene. As set forth in the previous office action (refer to Leonard and Griffiths), the phenotype of knockout mice is unpredictable. The specification disclosed phenotypes exhibited by some knockout mice that comprise a homozygous disruption in the glucocorticoid-induced receptor gene (pages 53-54); however, the specification does not teach a phenotype for mice comprising a heterozygous disruption in the glucocorticoid-induced receptor gene that differs from a wild-type mouse. The specification asserts that the claimed mice can be used for drug testing (pages 3-4 and 19-20), however, the specification fails to describe any phenotype for the mouse that correlates with a disease. The skilled artisan would not know how to use a transgenic knockout mouse that lacks a phenotype, particularly because the instant specification has not provided uses for such. Given the unpredictable nature if a phenotype that results from disruption of a nucleotide sequence, it

would have required undue experimentation for the skilled artisan to use the claimed heterozygous knockout mouse that lacks a phenotype.

Newly added claims 36-48 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement as applied to cancelled claims 5-12,17-29 and 33 for reasons of record as set forth on pages 5-6 of the previous office action mailed 03/12/2003.

Vas-Cath Inc. v. Mahurkar, 19USPQ2d 1111, clearly states that "applicant must convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession *of the invention.* The invention is, for purposes of the 'written description' inquiry, *whatever is now claimed.*" (See page 1117.) The specification does not "clearly allow persons of ordinary skill in the art to recognize that [he or she] invented what is claimed." (See *Vas-Cath* at page 1116).

The basis of the rejection that the specification fails to describe the broad genera of genes encompassed by the claims applies to the newly added claims 36-48. Applicant's arguments have been fully considered and are not found persuasive. Applicant argues that the newly added claims recite "the endogenous mouse glucocorticoid-induced receptor" (page 6, line 20), however claims 36, 42 and 47 recite "...disruption in endogenous mouse glucocorticoid-induced receptor gene" and are therefore not limiting to the mouse glucocorticoid-induced receptor described in the specification. Therefore, as stated on page 6 lines 9-15 of the previous office action, the claims can be read as being drawn to more than one mouse glucocorticoid-induced receptor gene and the specification only describes one mouse glucocorticoid-induced receptor gene.

In the instant case the mouse glucocorticoid-induced receptor genes encompassed by the claims lack a written description. The specification fails to describe what DNA molecules fall into this genus and it was unknown as of Applicants' effective filing date that any of these DNA molecules would have the property of encoding a glucocorticoid-induced receptor polypeptide having the same structural and functional properties as that encoded by SEQ ID NO:1. The claimed embodiments of glucocorticoid-induced receptor genes encompassed within the genus lack a written description. There is no evidence on the record of a relationship between the structures of the nucleotide sequences coding for a mouse glucocorticoid-induced receptor and the nucleotide sequence set forth by SEQ ID NO:1 that would provide any reliable information about the structure of DNA molecules within the genus. The claimed invention as a whole is not adequately described if the claims require essential or critical elements that are not adequately described in the specification and that is not conventional in the art as of applicants effective filing date. Possession may be shown by actual reduction to practice, clear depiction of the invention in a detailed drawing, or by describing the invention with sufficient relevant identifying characteristics such that a person skilled in the art would recognize that the inventor had possession of the claimed invention. Pfaff v. Wells Electronics, Inc., 48 USPQ2d 1641,1646 (1998).

With the exception of the sequence referred to above, the skilled artisan cannot envision the detailed chemical structure of the encompassed polynucleotides, and therefore conception is not achieved until reduction to practice has occurred regardless of the complexity or simplicity of the method of isolation. The skilled artisan cannot envision the detailed chemical structure of the encompassed nucleic acid molecules and therefore conception is not achieved until reduction

to practice has occurred, regardless of the complexity or simplicity of the method of isolation. Adequate written description requires more than a mere statement that it is part of the invention and reference to a potential method of isolating it. The nucleic acid itself is required. See *Fiers v. Revel*, 25 USPQ2d 1601 at 1606 (CAFC 1993) and *Amgen Inc. v. Chugai Pharmaceutical Co. Ltd.*, 18 USPQ2d 1016.

One cannot describe what one has not conceived. See *Fiddes v. Baird*, 30 USPQ2d 1481 at 1483. In *Fiddes*, claims directed to mammalian FGF's were found to be unpatentable due to lack of written description for that broad class. The specification provided only the bovine sequence.

In view of the above considerations one of skill in the art would not recognize that applicant was in possession of the necessary common features or attributes possessed by any member of the genus of genes encoding glucocorticoid-induced receptor. Therefore, only the glucocorticoid-induced receptor gene encompassed by **SEQ ID NO:1**, but not the full breadth of the claims meet the written description provision of 35 U.S.C. §112, first paragraph. University of California v. Eli Lilly and Co., 43 USPQ2d 1398, 1404, 1405 held that "to fulfill the written description requirement, a patent specification must describe an invention and do so in sufficient detail that one skilled in the art can clearly conclude that "the inventor invented the claimed invention".

Applicant is reminded that *Vas-Cath* makes clear that the written description provision of 35 U.S.C. §112 is severable from its enablement provision (see page 1115).

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

The previous rejection of claims 5-10 under 35 USC 103 is withdrawn.

Conclusion

No claim is allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Valarie Bertoglio whose telephone number is 703-305-5469. The examiner can normally be reached on Mon-Weds 6:00-2:30.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Deborah Reynolds can be reached on 703-305-4051. The fax phone number for the organization where this application or proceeding is assigned is (703) 872-9306.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is 703-308-1234.

PETER PARAS
PATENT EXAMINER

Valarie Bertoglio
Examiner
Art Unit 1632

Notice of References Cited	Application/Control No. 09/903,396	Applicant(s)/Patent Under Reexamination ALLEN, KEITH D.	
	Examiner Valarie Bertoglio	Art Unit 1632	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
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FOREIGN PATENT DOCUMENTS

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	Q					
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	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Liu, X. et al. , 2001, Biological Psychiatry, Vol. 49, pages 575-581.
	V	Mayorga, A.J et al. 2001, Psychopharmacology, Vol. 155, pages 110-112.
	W	Gass, P. et al. 2001, Physiology and Behavior, Vol. 73, pp. 811-825.
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Genetic Differences in the Tail-Suspension Test and Its Relationship to Imipramine Response among 11 Inbred Strains of Mice

Xiaoqing Liu and Howard K. Gershenfeld

Background: *The tail suspension test (TST) is a simple screening test for the behavioral effects of antidepressants in rodents. This experiment investigated the interindividual differences in responses to stressful situations measured by duration of immobility in the TST and the effects of imipramine (30 mg/kg intraperitoneally) in reducing immobility among 11 inbred strains of mice. The 11 inbred strains were 129S6/SvEvTac, A/J, AKR/J, Balb/cJ, C3H/HeJ, C57BL/6J, DBA/2J, FVB/NJ, NMRI, SencarA/PtJ, and SWR/J.*

Methods: *All mice underwent two trials of TST: 1) spontaneous, basal TST and 2) imipramine or saline TST. The duration of immobility was the trait measured during a 6-min test.*

Results: *In the four strains tested, female mice had longer duration of immobility than male mice in basal TST duration of immobility. For male mice (n = 11 strains), significant strain differences in immobility duration were found for both basal TST and imipramine response TST, with heritability estimates of .31 and .60, respectively. Immobility duration for the DBA/2J, FVB/NJ, and NMRI strains were significantly reduced by imipramine, relative to saline. Surprisingly, this reduction of immobility by imipramine was independent of the basal immobility.*

Conclusions: *These results suggest that the responses on basal TST and the imipramine-mediated responses on TST are mediated by separate genetic pathways.* Biol Psychiatry 2001;49:575–581 © *2001 Society of Biological Psychiatry*

Key Words: Behavioral despair, antidepressants, imipramine, inbred mice, tail suspension test, transgenic

Introduction

Depression and anxiety disorders are common psychiatric illnesses with remarkable interindividual differences in symptoms and drug responses, where some individuals completely respond and others demonstrate only a partial response. Although the individual differences in stress responses continue to be vigorously explored (Anisman and Zacharko 1992), the genetic sources of these differences in responsiveness largely remain a puzzle. Imipramine is well known to have both antidepressant and antianxiety activities (Cross-National Collaborative Panic Study, Second Phase Investigators 1992; Rickels et al 1993), but the mechanisms of individual differences in responsiveness to imipramine are incompletely understood. To investigate these separate phenomena of 1) genetic influences in "stress response" and 2) imipramine responsiveness in a simple mouse model, we selected the tail-suspension test (TST) (Chermat et al 1986; Nomura et al 1991; Porsolt et al 1987; Steru et al 1985, 1987; Thierry et al 1986). This paradigm is a well-validated test to screen for antidepressants in mice, while also modeling individual differences in stress responses. In the automated version, a mouse is suspended by its tail and a strain gauge measures the movements the mouse makes, calculating the duration of immobility below a given threshold. During a testing session, the mouse alternates between active attempts to escape and passive immobility. The duration of immobility has been inferred as an index of "behavioral despair," where longer durations of immobility imply a greater degree of behavioral despair. An evolutionary, ethological perspective construes the TST as a measure of coping or adaptation, reflecting an individual's strategic response when facing a problem of survival without solution (Thierry et al 1984). Antidepressants have an anti-immobility effect on the duration of immobility. The TST has been extensively validated as a screen for antidepressant activity with an impressive diversity of antidepressants (tricyclic antidepressants, selective serotonin reuptake inhibitors, buproprion and atypical antidepressants, monoamine oxidase

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inhibitors) and even electroconvulsive therapy (Perrault et al 1992; Steru et al 1985, 1987; Teste et al 1990, 1993). This paradigm has the additional virtues of being sensitive, with a high degree of pharmacologic predictive validity, and being atheoretical regarding the mechanism of antidepressant action. Most antidepressants maximally reduce the TST duration of immobility with doses less than those required for the forced swim test. For animal models of behavioral despair, the most investigated and validated antidepressant is the tricyclic imipramine. Imipramine has been repeatedly demonstrated to have dose-dependent anti-immobility effects in the TST, with maximal responses 30 min after a single intraperitoneal (IP) injection at 30 mg/kg (Steru et al 1987; Teste et al 1993; van der Heyden et al 1987). In the mouse, imipramine (20 mg/kg intravenously) has a plasma elimination half-life of 50 min (Dingell et al 1964).

Genetic differences have been found among inbred and outbred strains of mice measuring spontaneous duration of immobility in the TST. For example, the CD1 strain had a shorter duration of immobility relative to the NMRI strain (Vaugeois et al 1997). Although Trullas et al (1989) have previously compared the spontaneous TST scores for different inbred strains and showed marked differences among strains, their TST method relied upon manual observation and subjective judgements of the immobility, with less than ideal reliability and precision. Here, we used commercially available automated equipment and selected seven inbred mice strains from the major branches of the genealogical tree presented by Atchley and Fitch (1993), plus four other inbred strains: A/J, FVB/NJ, NMRI, and SencarA/PtJ. Secondly, studies within and between outbred strains on the TST have demonstrated differences in antidepressant response, perhaps modeling the variation in human responses to antidepressants. The outbred NMRI strain robustly responded to most antidepressants in the TST, whereas the CD1 strain was less responsive (Vaugeois et al 1997). From the widely dispersed distribution of an outbred mouse population's spontaneous TST immobility, stable individual differences could be defined for subgroups of "high" and "low" basal immobility mice (Vaugeois et al 1996). Interestingly, only the high basal immobility group was responsive to antidepressants. Hence, the TST imipramine response was inferred to be highly correlated with basal immobility. Furthermore, CD1 mice were bidirectionally selected and bred for high immobility versus low immobility in the basal TST. By the second generation, only the mice in the high immobility group were significantly responsive to imipramine. By a classical genetic design (i.e., varying the genotype), we re-examined this relationship between high basal TST immobility and imipramine response by surveying diverse mouse strains and examining the correlation between these traits.

As the mouse and human genomes become completely defined by sequencing, functional assays become critical in helping to characterize the role of unknown open reading frames. The objectives of this TST pilot study were to 1) optimize a high throughput automated paradigm, 2) extend the findings of genetic differences among inbred strains, and 3) provide baseline normative data of strain differences to permit the judicious selection of an inbred strain for mutagenesis studies and for breeding crosses of transgenic knockouts to an appropriate genetic background (Banbury Conference 1997; Crawley et al 1997). Since the available quantity of transgenic mice is often limiting, we aimed to establish a phenotyping procedure maximizing the information from each mouse, obtaining both baseline information and drug response data.

Methods and Materials

Animals

Eleven strains of inbred mice were tested in this experiment. Inbred A/J, AKR/J, Balb/cJ, C3H/HeJ, C57BL/6J, DBA/2J, FVB/NJ, SencarA/PtJ, and SWR/J mice were obtained from the Jackson Laboratory (Bar Harbor, ME) at 4–7 weeks of age. SencarA/PtJ mice were originally derived by crossing Charles River CD-1 mice with skin tumor-sensitive mice (Slaga 1986) and subsequently inbred (Hennings et al 1997). Male inbred NMRI and 129S6/SvEvTac mice were obtained from B & K Universal (Grimston, Hull, East Yorkshire, UK) and Taconic (Germantown, NY), respectively, at 6–7 weeks of age. All mice of the same strains were housed in a group of five with food and water freely available. The animals were maintained under a 12-hour light:dark cycle with lights on at 6:00 AM. They were identified by ear notching at 4–7 weeks age and allowed to adapt to their new housing conditions for at least 1 week before testing. All animals were brought to the testing room at least 1 hour before the commencement of each behavioral test, and remained in the same room throughout the test. Mice were tested individually during the light cycles between 11:00 AM and 5:00 PM at 8–9 weeks of age. Two days after naive mice were tested for basal TST the mice were tested for their response to imipramine. For each strain, the mice were randomly divided into two equally sized groups: an imipramine group and a saline group. All experiments followed the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the local institutional review committee.

Imipramine

Prior dose response studies consistently demonstrated 30 mg/kg of imipramine IP produced maximal TST responses without showing sedation (Steru et al 1985, 1987; Teste et al 1993; van der Heyden et al 1987). Our preliminary dose response experiments confirmed this on the inbred 129S6/SvEvTac and the outbred ICR strains. Indeed, 30 mg/kg of imipramine was the optimal dosage, whereas the dose of 60 mg/kg was sedating in

the ICR strain (data not shown). Therefore, a fixed dose of imipramine (30 mg/kg) was selected. Imipramine HCl (RBI/Sigma, Natick, MA) was dissolved in pyrogen-free saline (0.9% NaCl) and prepared on the day of the experiment. Imipramine or saline was administered IP 30 min before TST testing, in a volume of 0.1 mL/g body weight.

Tail Suspension Test

Automated TST devices (Med Associates, St. Albans, VT) were used to measure the duration (sec) of immobility in the TST. Mice were suspended by the tail with tape (Scotch Super Strength mailing tape, 3M Corp, St. Paul) to an aluminum bar connected to a strain gauge. The strain gauge detected movements of the mouse and transmitted them to a central unit. The total duration of immobility was automatically calculated as the time the force of the mouse's movements was below a preset threshold criterion (i.e., immobile and not struggling) during a 6-min TST test. The most discriminating settings for detecting immobility were determined empirically. The device was configured with the following settings: time constant = 0.25, gain = 4, threshold 1 = 3, and resolution = 200 msec. Whenever the mouse's movements were lower than our threshold 1 (<3) for 200 msec, the duration of the immobility accumulated.

Imipramine Levels

Drug concentrations of imipramine and its active metabolite desipramine were determined in brain tissue by gas chromatographic mass spectrometry as previously described (Belvedere et al 1975). Mice were injected with imipramine (30 mg/kg IP) and then 30 min later the mice were euthanized with CO_2. The brain was quickly removed, weighed, and frozen at −80°. Imipramine and desipramine levels were determined on individual brain samples in duplicate for NMRI and 129S6/SvEvTac mice (n = 5/strain). The NMRI and 129S6/SvEvTac strains were selected as representatives of mice that were highly imipramine responsive versus much less responsive, respectively. For each mouse, the sum of the imipramine and desipramine brain levels was the dependent variable.

Statistics

One-way analyses of variance (ANOVAs), followed by post hoc tests (Student-Newman-Keuls test and Tukey compromise), tested difference of duration of immobility for basal TST among the 11 strains (male mice only). The imipramine response was determined as the percentage change in immobility after the administration of imipramine relative to the saline group in each strain. A correlation was performed between immobility of basal TST and the change of immobility due to imipramine for the 11 strains, using the means of inbred strains. The strain means of the brain drug concentration were compared by a Mann-Whitney U test. Narrow sense trait heritabilities (i.e., the proportion of variance due to additive genetic variance) were determined by comparing the between-strain variance to the total variance. Inbred strains are isogenic (i.e., genetically identical). Hence, between-strain variance provided a measure of additive genetic variation (V_A), whereas within-strain variance represents environmental variability (V_E). An estimate of heritability (h^2) for each trait (basal immobility and imipramine immobility) was obtained using the formula: $h^2 = V_A/(V_A + V_E)$ (Falconer and Mackay 1996). This measure provides an estimate of the trait variance attributable to genetic factors as opposed to environmental factors. Gender differences on basal TST were analyzed for four strains (129S6/SvEvTac, A/J, C57BL/6J, and NMRI) using two-way ANOVA with strain and gender as main factors on basal TST.

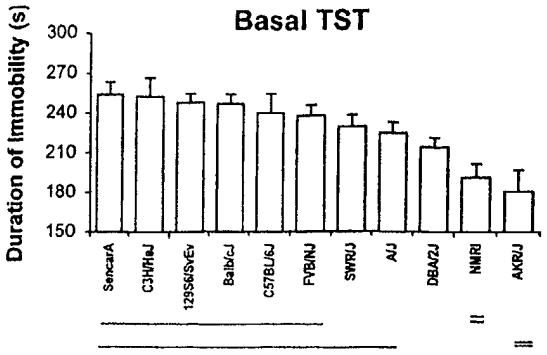

Figure 1. Strain distribution of basal immobility across 11 inbred strains (n = 10 for each strain), showing mean (± SEM) duration of immobility. Bars below the figure designate strains that significantly differ (Newman-Keuls, $p < .05$). TST, tail suspension test.

Results

Strain Distribution of Basal Immobility

The mean durations of immobility measured in the TST across 11 inbred strains (males only, n = 10 for each strain) of naive mice are shown in Figure 1. This figure highlights the continuous distribution pattern of basal TST immobility. Significant strain differences were detected in spontaneous immobility [$F(10, 98) = 5.4, p < .001$]. Post hoc comparisons (Student-Newman-Keuls) indicated that the NMRI strain's duration of immobility was significantly shorter than those of the SencarA/PtJ, C3H/HeJ, 129S6/SvEvTac, Balb/cJ, C57BL/6J, and FVB/NJ mice, whereas that for the AKR/J strain was significantly shorter than all the strains except DBA/2J and NMRI. Immobilities for other strains did not differ significantly from each other.

Imipramine Response to TST

Table 1 compares the durations of immobility following treatment with imipramine (30 mg/kg IP) and saline control. Imipramine demonstrated a robust anti-immobility effect for three strains—namely, DBA/2J [$t(8) = 4.63$, $p < .01$], FVB/NJ [$t(8) = 3.5, p < .01$], and NMRI [$t(8) = 3.19$, $p < .05$]. Figure 2 shows the strain distribution in

Table 1. Duration of Immobility (6-Min Period) in the Tail Suspension Test for the 11 Inbred Strains of Mice

Strain	Imipramine immobility	Vehicle immobility
129S6/SvEvTac	249 (11)	269 (34)
A/J	208 (17)	233 (28)
AKR/J	215 (24)	165 (62)
Balb/cJ	214 (50)	275 (23)
C3H/HeJ	227 (66)	279 (36)
C57BL/6J	276 (17)	302 (22)
DBA/2J	180 (33)[a]	266 (27)
FVB/NJ	190 (32)[a]	247 (18)
NMRI	155 (50)[b]	240 (33)
SencarA/PtJ	264 (16)	278 (28)
SWR/J	221 (13)	229 (14)

Values are means (±SDs) for tail-suspension test following injection of saline or imipramine (30 mg/kg, intraperitoneally).

[a] $p < .01$, significant difference between saline and imipramine groups within each strain.

[b] $p < .05$, significant difference between saline and imipramine groups within each strain.

response to imipramine (percentage change in immobility) relative to saline control. The response to imipramine significantly differed among strains [$F(10, 44) = 8.50$, $p < .0001$]. The distribution pattern of response to imipramine is continuous across strains, with a maximal response for NMRI. For the AKR strain, imipramine actually increased the duration of immobility, relative to all other strains (Student-Newman-Keuls, $p < .05$).

To examine the relationship between the duration of basal immobility and the imipramine responses, a correlation was performed between the means of basal immobility and the percentage change in immobility for each strain. The correlation coefficient was not significant ($r = .06$). Likewise, a Spearman correlation on the ranked data was not significant either ($\rho = .15$).

Heritability is a statistic estimating the extent to which

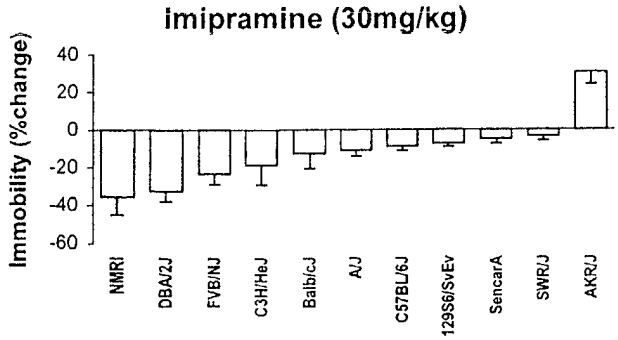

Figure 2. Strain distribution of imipramine response across 11 inbred strains, showing mean (± SEM) percentage change in duration of immobility. Percent change in duration of immobility was calculated by dividing the imipramine (30 mg/kg intraperitoneally) immobility by the saline immobility and multiplying by 100.

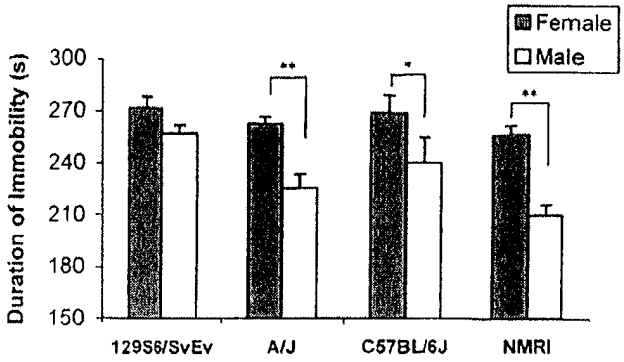

Figure 3. Gender differences in basal immobility across four strains are compared, showing mean (± SEM) duration of immobility. Asterisks indicate significant differences between genders within strains (*$p < .05$, **$p < .01$).

genetic variation contributes to the total population (phenotypic) variation, varying from none to completely (i.e., from 0 to 1). By prudent selection of mice from the major branches of the genealogical tree, these inbred strains provide a reasonable representation of genetic diversity for heritability estimates. The narrow sense trait heritabilities for basal immobility and imipramine-induced reduction in immobility were estimated as .31 and .60, respectively.

Finally, we wanted to examine whether the differential strain responses to imipramine (i.e., NMRI as an imipramine responder vs. 129S6/SvEvTac as a minimal responder) were pharmacokinetic. The brain drug levels (viz., the sum of imipramine and desipramine) did not differ significantly between strains ($U = 13.0$, $p = .92$), with mean values (± SD) of 12,274 ± 5010 ng/g for NMRI and 14,961 ± 3121 ng/g for 129S6/SvEvTac. Hence, this differential response to imipramine is more likely to be pharmacodynamic rather than pharmacokinetic.

Gender Differences in Basal Immobility

An overall gender difference was found across four strains (129S6/SvEvTac, A/J, C57BL/6J, and NMRI) on basal TST (Figure 3), with longer immobility for female mice (mean difference = 33 sec). A two-way ANOVA on basal immobility yielded a main gender effect [$F(1,121) = 34.86$, $p < .0001$] and an insignificant interaction effect [$F(3,121) = 1.97$, $p = .12$], supporting this observation. For the A/J, C57BL/6J, and NMRI strains, there were significant differences, indicated by the post hoc comparisons, between male and female mice (Figure 3).

Discussion

We consider TST behavior to be a simple model for understanding strain differences in "reactivity to stress" and a mini-model of imipramine responsivity. We hypoth-

esized that the basal TST in mice may model genetic differences in liability for "general distress," which is a risk factor for psychiatric disorders (Kendler et al 1992). These experiments demonstrate the reliability of an automated TST paradigm in male mice and provide a set of normative data for research in pharmacology and for mutagenesis studies (Brown and Nolan 1998; Schimenti and Bucan 1998). The results also permit the judicious selection of inbred strains and genetic backgrounds for sensitively detecting the effects of transgenic mouse lines (Banbury Conference 1997). The significant natural variation among inbred mouse strains in their basal TST confirms and extends prior findings of individual differences among mouse populations (Trullas et al 1989; Vaugeois et al 1997). Likewise, the genetic estimate of heritability for basal TST performance of .31 is consistent with the estimated heritability of other behavioral traits, personality traits, and psychiatric illnesses (Plomin et al 1994).

A second finding is the marked natural variation and high heritability estimate (.6) in imipramine responsivity among strains as measured by the reduction in the duration of immobility compared to a saline control. To our knowledge, this is the first report of inbred strains (viz., DBA/2J, FVB/NJ, and NMRI) exhibiting significant TST immobility reduction comparable to prior findings for the robust effect of imipramine in outbred strains (Nomura et al 1991; van der Heyden et al 1987; Vaugeois et al 1997). Two genetic mechanisms for a marked response versus a minimal response to imipramine among strains were considered—namely, pharmacodynamic versus pharmacokinetic explanations. Strains of mice may differ in their disposition of a drug, with variations in absorption, distribution, and metabolism (Sallee and Pollock 1990). To examine this hypothesis, we tested two strains of mice with marked differences in their behavioral response to imipramine for brain levels of imipramine and desipramine after a fixed dose (30 mg/kg IP). The results showed no significant difference in the drug concentrations between strains. Hence, for these two strains the differential response to imipramine is more likely to be pharmacodynamic, such as differences in receptor density, receptor affinity, or downstream, postreceptor transduction pathways (Bonhomme and Esposito 1998; Popoli et al 2000). However, we have no evidence to generalize our findings beyond these two strains.

Further understanding of the TST paradigm stems from the near zero correlation of basal immobility with the imipramine-induced reduction of immobility. Unlike prior studies in outbred strains suggesting a relationship between basal immobility and imipramine response (Vaugeois et al 1996, 1997), this panel of inbred strains indicates a genetic dissociation between factors influencing basal immobility and imipramine response. These findings suggest separate, distinct genetic architectures for basal immobility and for imipramine-induced reduction of immobility. Secondly, this near zero correlation suggests that the anti-immobility effect of antidepressant response does not depend on initial values (i.e., disagreement with the "law of initial value") (Jin 1992). Finally, concerns over ceiling or floor effects based on a strain's baseline duration immobility become less likely.

These results of genetic variation further validate this TST model (Porsolt 2000), and the findings dovetail with other rodent models of stress and depression displaying genetic differences in behavior (Anisman and Zacharko 1992; Overstreet et al 1995; Porsolt et al 1978; Shanks and Anisman 1988, 1989; Shanks et al 1990; Wieland et al 1986). For example, the Wistar-Kyoto rat strain was defined as subsensitive to imipramine relative to Brown–Norway, Fischer 344, and Sprague-Dawley (Lahmame and Armario 1996). Clinically, patient populations with depressive or anxiety disorders also show a similar variation in responsivity versus refractiveness to imipramine treatment, even at adequate drug levels (Marks 1987; Stewart et al 1998).

For the four strains tested for gender effects, the female mice responded to the TST with longer duration of immobility than male mice. This gender difference on basal immobility agrees with prior work showing that ovarian hormones increase the duration of immobility in ovariectomized mice and restore duration of immobility to baseline levels in the TST (Bernardi et al 1989). A further interesting question remains regarding how much of the ovarian hormone effect is an "organizational" and permanent effect on brain development versus a more transient, reversible "activational" effect (McEwen et al 1991; Patchev and Almeida 1998). Perhaps this gender difference is important, mirroring epidemiologic surveys finding a female predominance to major depression, generalized anxiety disorder, agoraphobia, and panic disorder (Marks 1987; Weissman et al 1997; Weissman and Olfson 1995).

For these experiments, we were only able to statistically detect robust imipramine responses with an effect size of 1.7, and a caveat was the limited statistical power for detecting subtle effects. A second limitation regards a general criticism of the TST as a primary screen for antidepressants, since it only examines the "acute" effects of imipramine. Indeed, most antidepressants are prescribed chronically and have a lag period before clinical response of 2–4 weeks. However, this TST claims only to be a mini-model for aspects of depression and a primary screen for a broad range of antidepressants (Porsolt 2000).

In conclusion, this paradigm taps innate behavior to provide an atheoretic, sensitive, pharmacologically vali-

dated, high throughput mini-model of depression in mice. These experiments highlight the importance of heritable strain and gender differences in the TST model. The recognition of the independence of basal immobility from the anti-immobility effect of imipramine may permit more precise selective breeding experiments, yielding improved model systems for antidepressant screenings. The optimization of an automated, functional screening assay may facilitate transgenic mouse studies and mutagenesis screens on the appropriate genetic background strain. As neuronal and neuroendocrine genes become accessible through genome sequencing and in transgenics, the biological processes underpinning these phenomena can be further dissected. We anticipate this work will advance the identification and validation of genes affecting these complex behaviors as mini-models relevant to psychiatric illness.

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RAPID COMMUNICATION

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Limitations on the use of the C57BL/6 mouse in the tail suspension test

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Abstract *Rationale*: The C57BL/6 is one of the most widely used mouse strains in behavioral, pharmacological, and genetic research but little is known about their response on tests for antidepressant drugs. *Objectives*: The behavior of C57BL/6 mice, and mice from other strains, was examined in the tail suspension test (TST), a common behavioral test used for the screening of antidepressant compounds. *Methods*: C57BL/6J mice from the Jackson Laboratory, C57BL/6N mice from Harlan, A/J, 129-SV-ter and DBA/2 mice were tested under baseline conditions in the TST. *Results*: The majority of the C57BL/6 mice from the Jackson Laboratory tested in this paradigm (70%) climbed up their tails during the 6-min test session. C57BL/6 mice obtained from Harlan (35%) also demonstrated this climbing behavior, suggesting that it is not specific to mice from a particular supplier. Other strains (A/J 18%), 129-SV-ter (0%) and DBA/2 (0%) mice) showed less propensity for tail climbing. *Conclusions*: The occurrence of this behavior is an important consideration when testing antidepressant drugs or the effects of stress using the TST with inbred mouse strains, especially those from the C57BL/6 strain.

Keywords Tail suspension test · Depression · Antidepressant · C57BL/6 · Mouse

Introduction

The C57BL/6 is one of the most widely used mouse strains in behavioral pharmacology research (for review, see Crawley et al. 1997). It has also been used as a background strain for the development of genetic knockout mice (e.g. Baik et al. 1995) and has been utilized in the development of recombinant inbred lines of mice for genetics research (Gora-Maslak et al. 1991). Since interactions with genetic background are an important consideration in the choice of strain for behavioral research, it is particularly relevant to consider any limitations of the use of a given strain in commonly used behavioral assays. C57BL/6 mice have been successfully used in a number of behavioral paradigms to characterize psychotropic agents, including but not limited to models of learning and memory, schizophrenia, drug abuse, anxiety and motor function (Crawley et al. 1997). Our laboratory has previously demonstrated that C57BL/6 mice can also be used in the forced swim test for antidepressant activity (Dalvi and Lucki 1999). However, we now report that the use of this strain in the tail suspension test for antidepressant activity may be problematic, due to a propensity for these animals to climb up their tails during the testing session.

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Materials and methods

Male C57BL/6 mice (8–10 weeks of age) were obtained from the Jackson Laboratories (C57BL/6J, Bar Harbor, Maine, USA) and Harlan (C57BL/6NHsd, Indianapolis, Ind., USA) and housed in a colony room maintained at 21°C under a 12-h light-dark cycle (lights on at 0700 hours) for 2 weeks prior to testing. Food and water were freely available. C57BL/6 mice (n=20 per supplier) were tested in the tail suspension test and then retested under the same conditions 1 week later. For comparison with the C57BL/6 strains, other strains of mice [129-Sv-ter (n=15; University of Pennsylvania), DBA/2 (n=10; Jackson Laboratories) and A/J (n=11; Jackson Laboratories)] were tested under similar conditions as part of a drug-testing experiment. The tail suspension test was a modified version of that validated for NMRI mice by Steru and colleagues (1985). Mice were transported a short distance from the holding facility to the testing room and left there undisturbed for at least 3 h. Subjects were each given an intraperitoneal injection of 0.9% saline (10.0 ml/kg). Thirty minutes after injection, a standard interval in drug-testing experiments, mice were individually suspended by the tail to a horizontal ring-stand bar (distance from floor=35 cm) using adhesive tape (distance from tip of tail=2 cm). Typically, mice demonstrated several escape-oriented

behaviors interspersed with bouts of immobility of increasing length as the session progressed. A 6-min test session was employed which was videotaped. The number of animals that climbed their tails up to the horizontal bar was recorded, along with their latency (in seconds) to do so. Further experiments using smaller numbers of subjects were conducted to determine whether certain procedural manipulations could overcome the tail-climbing problem.

Results

A large proportion (14 out of 20 or 70%) of the C57BL/6J mice obtained from the Jackson Laboratory climbed their tails at some point during the 6-min tail suspension test session (see photograph in Fig. 1). The mean latency of the mice to climb their tails was 18.4 s (SD=8.8, minus one outlier with latency=160 s). Upon retesting under the same conditions 1 week later, the same proportion of mice (70%) from this group climbed their tails. In a separate group of eight C57BL/6J mice obtained from the Jackson Laboratory, the same proportion (three out of four or 75%) climbed their tails regardless of whether the tail was taped 2 cm from the tip or 2 cm from the base. A lower proportion of C57BL/6 mice obtained from Harlan (C57BL/6NHsd) climbed their tails (seven out of 20 or 35%) and a similar proportion from this group (eight out of 20 or 40%) climbed their tails upon retesting 1 week later. The mean latency of the C57BL/6 mice from Harlan to climb their tails was 40.2 s (SD=18.5, minus one outlier with latency=245 s). In studies conducted by this laboratory using the same TST procedure but with other mouse strains (unpublished data), two of 11 A/J mice, none of 15 129-SV-ter mice and none of ten DBA/2 mice demonstrated this behavior.

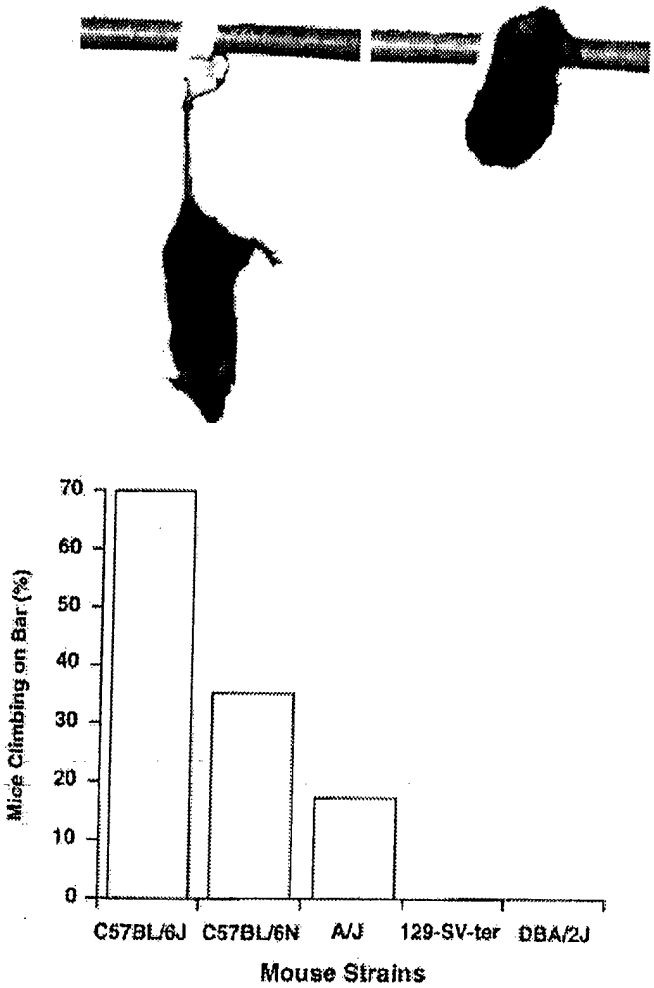

Fig. 1 A photograph showing the tail-climbing response of C57BL/6J mice in the tail suspension test is shown at the *top* of the figure. The animal on the *left* shows a C57BL/6J mouse fully extended below the retaining bar showing the typical posture of behavioral immobility. The animal on the *right* shows a C57BL/6J mouse that has climbed up its tail to grasp the retaining bar. The percentages of mice tested showing tail-climbing behavior are illustrated in the graph at the *bottom* of the figure. The numbers of animals tested were (in *parentheses*): C57BL/6J (20), C57BL/6N (20), A/J (11), 129-SV-ter (15) and DBA/2 (10)

Discussion

The current data demonstrates that a substantial proportion of C57BL/6 mice may fail to produce valid data in the tail suspension test due to their tendency to climb their tails up to the horizontal bar, as suggested by Dalvi and Lucki (1999). The latency for the mice to climb their tails is relatively short, suggesting that simply decreasing the length of the test session will not eliminate the problem. Furthermore, the tail-climbing behavior recurs upon subsequent exposure to the test, suggesting that it is a reliable behavioral phenomenon. The tail climbing occurs regardless of the position at which the tail is taped, although it is uncertain whether other procedural variations would decrease the occurrence of the behavior. C57BL/6 mice obtained from Harlan also demonstrated the tail-climbing behavior, suggesting that it is not specific to a particular animal supplier. The tail-climbing response may be particular problematic when an automated version of the tail suspension test is being used if the behavior of the animals within an enclosure is not being directly observed because investigators may not be aware of whether this behavior is influencing their results. This phenomenon should be considered when planning experiments to characterize potential antidepressant compounds in mice using the TST, as well as in the choice of mouse strain for the generation and testing of mutant knockout mice with murine antidepressant tests (Dalvi and Lucki 1999).

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Mice with targeted mutations of glucocorticoid and mineralocorticoid receptors: Models for depression and anxiety?

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Abstract

Impaired corticosteroid receptor signaling is a key mechanism in the pathogenesis of stress-related psychiatric disorders such as depression and anxiety. Since in vivo expression and functional studies of corticosteroid receptors are not feasible in the human central nervous system, such analyses have to be done in animal models. Transgenic mice with mutations of corticosteroid receptors are promising tools, which allow us to investigate the role of these proteins in the pathogenesis of symptoms characteristic for depression and anxiety. This review summarizes the neuroendocrinological and behavioral findings that have been obtained in six different mouse strains with specific mutations that influence the expression or the function of the glucocorticoid or the mineralocorticoid receptor (MR). The analyses of these mice helped to define molecular concepts of how corticosteroid receptors regulate the activity of the hypothalamic–pituitary–adrenal (HPA) system. Furthermore, some of these mutant mice exhibited characteristic alterations in behavioral tests for anxiety and despair. However, so far, none of the mouse strains described here can be viewed as an animal model of a specific psychiatric disease defined by common diagnostic criteria. Using high throughput technologies for the identification of genes regulated by glucocorticoid receptor (GR) and MR in brain areas responsible for specific symptoms of stress-related disorders will yield potential new drug targets for the treatment of depression and anxiety.

Keywords: Depression; Anxiety; Animal models; Gene targeting; Transgenic mice; Glucocorticoid receptor; Mineralocorticoid receptor; Transcription factors

1. Introduction

Dysregulation and dysfunction of corticosteroid receptors have been implicated in the pathogenesis of stress-related psychiatric disorders such as depression and anxiety (for review, see Refs. [1–8]). A central question is whether the disturbed corticosteroid receptors are a cause or a consequence of affective disorders. Clinical studies have convincingly shown a hyperactivity of the hypothalamic–pituitary–adrenal (HPA) system, leading to elevated plasma cortisol levels in many patients with major depression [9–11]. In contrast, HPA system alterations in patients with anxiety disorders are more subtle and inconsistent [12,13]. In patients with major depression, diminished corticosteroid receptor expression or functioning can be seen as a causative factor for a deficient feedback action of cortisol and can explain their increased HPA function and stress sensitivity. An alternative view sees the primary cause of the HPA dysregulation in an upregulation of hypothalamic corticotropin-releasing hormone (CRH), which secondarily leads to corticosteroid receptor downregulation in the limbic system and the hypothalamus and so perpetuates the disease state. The monoaminergic concept of depressive disorders regards the changes of the HPA system as secondary to a primary disturbance of serotonergic and noradrenergic brainstem neurons and their widespread connections to higher brain centers including cortex, limbic system and hypothalamus [14].

Studies of expression and function of corticosteroid receptors in the human central nervous system (CNS) have

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limitations due to the intracellular location of the receptors and a lack of suitable probes for their detection in vivo. Therefore, such analyses have to be done in animal models, e.g., in rodent models for depression and anxiety [15–17]. Molecular, endocrinological and structural alterations detected in such models can represent pathogenic mechanisms of the disorder. Alternatively, they may only be consequences of the experimental protocol. For example, the so-called 'depressive behavior' in mice is induced by stress exposure, which by itself can modulate corticosteroid receptor expression or function. One way to support a causal relationship between the modelled disease and the molecular changes observed is the successful application of pharmacotherapy with reversal of both the molecular and the behavioral alterations.

Another approach to correlate altered steroid signaling with behavioral changes involves the use of transgenic mouse models. Here, the reduction of corticosteroid receptor function is genetically induced [18–20]. When tested in behavioral paradigms, it is possible to determine whether and how steroid receptors participate in the development of specific symptoms of affective disorders. For example, one can determine whether the absence or the alteration of a corticosteroid receptor renders animals more prone or more resistant to develop features of depression and anxiety. Current techniques allow conditional gene disruption in specific anatomical regions, which in the best case are inducible at chosen timepoints [21,22]. Thus, a closer correlation between regional and temporal expression of corticosteroid receptors and their effects on CNS structure and function can be drawn, e.g., by doing electrophysiological and behavioral experiments. The present review outlines a transgenic approach that will help to define the role of two important corticosteroid receptors in depression and anxiety, i.e., the mineralocorticoid and glucocorticoid receptor (GR).

2. Molecular and functional properties of corticosteroid receptors

Molecular studies have so far revealed two corticosteroid receptor subtypes: type 1 or mineralocorticoid receptor (MR) and type 2 or GR [23,24]. In the CNS, the MR binds corticosterone with a tenfold higher affinity than the GR. Thus, in vivo, the MR is largely occupied by basal corticosterone levels, whereas the GR only becomes substantially occupied at the circadian maximum or during stress (for review, see Ref. [25]). This has led to the hypothesis that in areas where both receptor types are co-expressed, the MR mediates a baseline 'tonic' action of corticosteroids involving maintenance of homeostasis, as opposed to the 'phasic' actions of the GR that require increased hormone levels and are aimed at restoring stress-induced disturbances of homeostasis [2,26]. In addition to different ligand-binding affinities, differential expression patterns of both receptor subtypes in various neuronal cell populations add another level of complexity to the regulatory potential of the corticosteroid receptor system. Thus, high levels of GR are found throughout all major CNS neuronal populations [27,28]. In contrast, the MR is rather selectively and strongly expressed in the limbic system, e.g., in hippocampus, septum and amygdala complex [28–30].

Corticosteroid receptors function as transcription factors [18,31]. Corticosteroids act by binding to and activating their intracellular receptors, which then translocate to the cell nucleus (Fig. 1). There they attach as dimers to specific palindromic pentadecamer DNA sequences (glucocorticoid response elements, GREs) in the regulatory region of target genes, which in turn affects transcription (Fig. 1). For the GR, both positive and negative regulations of target genes have been described. Activation of transcription occurs via well-conserved GREs, while negative regulation (transrepression) is mediated by less conserved negative GREs (nGREs). Although the DNA-binding domains of the GR and the MR are nearly identical, the MR exhibits less transcriptional activity at GREs in in vitro transfection assays. Furthermore, the synergizing effect of multiple GREs with GR-activated transcription is not observed with MR activation, probably due to the limited homology of N-terminal sequences [32,33]. More recently, a second major mechanism by which the GR controls transcription was identified. GR monomers can repress as well as activate gene transcription via protein–protein interactions with other transcription factors, such as CREB, AP-1 and Stat5 (Fig. 1) [34–36]. Such protein–protein interactions have so far not been reported for the MR. Apart from nuclear actions, glucocorticoids exert rapid effects in the CNS within seconds or minutes, influencing neuronal physiology and function [37]. These early actions are thought to be mediated through specific receptors at the neuronal cytoplasma membrane [38]. Crucial to this hypothesis, however, will be the cloning of plasma membrane steroid receptors and their linkage to second messenger pathways.

Activation of corticosteroid receptors in neurons can influence diverse cellular processes such as energy metabolism, signal transduction and even structural plasticity. Functional consequences include the control of excitability in limbic brain regions, in particular in the hippocampus, where MR and GR are co-expressed. Predominant MR activation at the nadir of circadian corticosterone levels is associated with maintenance of hippocampal excitability. Thereby steady excitatory inputs to the hippocampal CA1 area result in considerable excitatory hippocampal output. In contrast, additional GR activation after acute stress or at diurnal peak generally depresses hippocampal output (for further reading, see the following reviews: Refs. [2,25,39,40]). These corticosteroid receptor-mediated effects at the cellular level have consequences for functional processes in which the hippocampal formation plays an essential role, e.g., in the neuroendocrine regulation of the

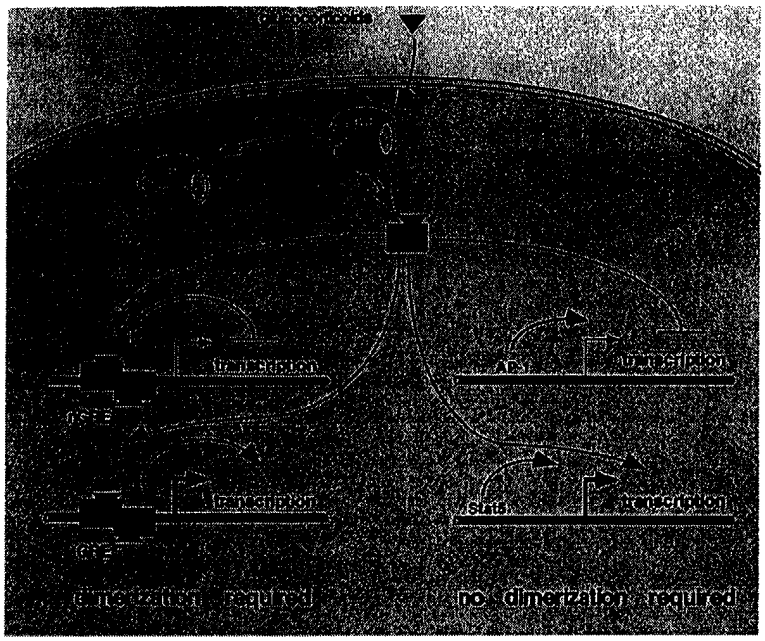

Fig. 1. GR is a multifaceted transcription factor. Glucocorticoid binding to the GR complex causes dissociation of a multiprotein complex (mpc) from the GR and effects a switch from the inactive to the active receptor conformation. Activated GR can modulate transcription by different modes: The binding to positive or negative regulatory DNA elements (GREs and nGREs, respectively) requires dimer formation and results in transactivation or transrepression of target genes. However, activated GR can also regulate as monomer the transcriptional activity of target genes. This occurs via protein–protein interactions with other transcription factors such as AP-1 or Stat5.

HPA system, and also in behavior. Thus, glucocorticoids influence perception and spontaneous behavior as well as other higher functions such as learning and memory (for review, see Ref. [2]). MR- and GR-mediated effects can be discriminated. Hippocampal MRs are responsible for corticosterone effects on appraisal of information and response selection, whereas GRs promote processes underlying consolidation of acquired information [41,42]. Both, MR- and GR-mediated effects on information processing facilitate behavioral adaptation, and thereby promote the inhibitory control exerted by higher brain centers over the HPA system activity.

3. HPA system regulation

The regulation of glucocorticoid synthesis and release is tightly controlled by the HPA system (Fig. 2) [43]. Stress and other stimuli induce synthesis and release of CRH and arginine–vasopressin (AVP) from neurons localized in the paraventricular nucleus (PVN) of the hypothalamus. This leads to increased synthesis and secretion of adrenocorticotropin hormone (ACTH) from the anterior lobe of the pituitary and thereby stimulates glucocorticoid production and release in the adrenal cortex. Cortisol is the principal circulating glucocorticoid in man, while corticosterone has this function in rodents. In a negative feedback loop, elevated plasma glucocorticoid concentrations cause repression of CRH and ACTH on the level of transcription and secretion, leading to homeostasis of glucocorticoid levels. Both the GR and the MR have been postulated to be involved in the feedback control of the HPA system [44]. The MR is thought to play a role in the regulation of circadian fluctuations of corticosteroids. The GR is believed to be important for the termination of the corticosteroid response when endogenous glucocorticoids are high due to stress or at circadian peak [44]. Diurnal mammals such as humans show high corticosteroid levels in the morning and low levels in the evening; for nocturnal rodents, it is vice versa.

4. Corticosteroid receptor function in depression and anxiety

Abnormalities in the HPA system are so far the most consistently demonstrated biological markers in depressive illness [45]. A significant number of patients with major depression show increased cortisol levels in plasma, cerebrospinal fluid, saliva and urine due to adrenal hypersecretion (Table 1). Furthermore, nonsuppression of ACTH and cortisol following dexamethasone administration, blunted ACTH responses to a CRH challenge, increased cortisol responses to ACTH and adrenal hypertrophy has been described (Table 1; for review, see Ref. [8]). The most sensitive neuroendocrine function test to detect HPA system dysregulation in patients combines the suppressive effect of dexamethasone with the stimulatory potency of

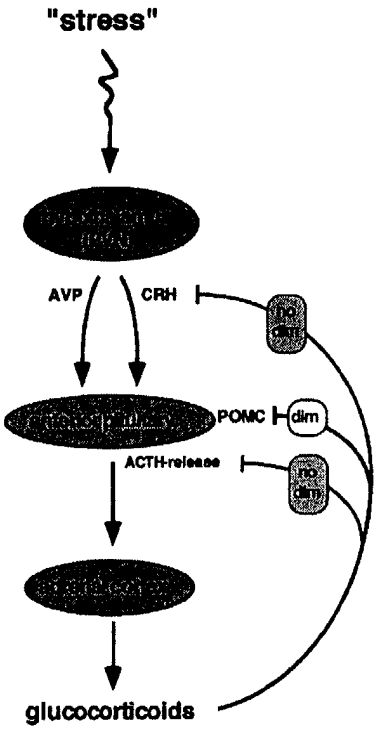

Fig. 2. GR-dependent regulation of the HPA system. In response to stress and under the control of higher brain regions such as hippocampus and amygdala complex, neurons of the PVN of the hypothalamus synthesize and secrete the neuropeptides, CRH and AVP. These hormones stimulate the production of ACTH in the anterior pituitary. Pituitary ACTH release results in synthesis and release of adrenocortical glucocorticoids. Their levels are tightly controlled by several negative feedback loops on the production and release of CRH and ACTH. Only the synthesis of the ACTH precursor, POMC, is under control of GR dimers (dim = dimerization required), whereas CRH synthesis and ACTH release are controlled by GR monomers (no dim = no dimerization required).

CRH and has been called dex/CRH test [46–48]. Whereas the CRH-elicited ACTH response is blunted in depressive patients, dexamethasone pretreatment produces the opposite effect and paradoxically enhances ACTH and cortisol release following CRH. The dex/CRH test creates the situation of a pharmacological (partial and transient) adrenalectomy in which hypothalamic CRH, and in particular vasopressin, expression is increased due to a decrease in plasma cortisol [8]. Vasopressin is thought to synergize with the intravenously applied CRH, overriding dexamethasone suppression at human pituitary corticotrophs. During depressive episodes, the patients' plasma cortisol levels often remain elevated throughout the day with a flattening of the normal diurnal variation. Normalization of the hyperactive HPA system occurs with successful pharmacotherapy with tricyclic antidepressants. Therefore, the hypothesis has been put forward, that dysfunctions of limbic structures and hypothalamic nuclei result in hypersecretion of CRH and in turn in hyperactivity of the HPA system. According to this concept, a primary disturbance in brain corticosteroid receptors (GR and MR in the hippocampus, GR in the hypothalamus) leads to disinhibition of CRH expression and secretion [20,45]. Alternatively, a primarily increased drive of CRH and vasopressin expression could result in excess levels of ACTH and cortisol, with a secondary decrease of corticosteroid receptor expression, capacity and function.

The release of CRH from neurons of the hypothalamic PVN is, in addition, under the control of a wide array of neurotransmitters, e.g., noradrenergic inputs from the locus coeruleus and serotonergic projections from the raphe nuclei. The fact that tricyclic antidepressants block the reuptake of these monoamines provides one of the cornerstones of the monoamine hypothesis of depressive illness. The monoamine theories of depression regard the defects in monoaminergic function as primary and abnormalities in the HPA system as secondary. However, several findings raise the possibility that central monoamine abnormalities can be induced by glucocorticoids. Adrenalectomy, i.e.,

Table 1
HPA system dysregulation and behavioral symptoms in mice with targeted mutations of GR and MR

	Baseline HPA system				Challenged HPA system			Behavior		
	Hypothalamic[a] CRH	Pituitary POMC/ACTH	Plasma ACTH	Plasma CORT	Stress-induced[a] ACTH	Stress-induced[a] CORT	dex/CRH test	Anxiety	Despair	Locomotion
Human depression	↑	n.d.	↑	↑	↓	↑	↑	↑	↑	
Human panic disorder	↔	n.d.	↔	↔	↓	↔	(↑)	↑	↑	
(1) Tg mice with ↓ GR	↓	↔	↔	↔	↑	↓	↑	↓	↓	↔
(2) GR^{null} mice	↑	↑	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
(3) GR^{NesCre} mice	↑	↑	↓	↑	↓	↔	n.d.	↓	↓	↔
(4) GR^{dim} mice	↔	↑	↔	↑	n.d.	n.d.	n.d.	↔	↔	↔
(5) Tg mice with ↑ GR	↓	↓	↑	↓	↑	↓	n.d.	n.d.	n.d.	n.d.
(6) MR −/− mice	↑	↑	↓	↑	n.d.	n.d.	n.d.	↑	n.d.	n.d.

Changes in human depression (↑ or ↓) were derived from comparisons with healthy control subjects. Changes in mutant mice were derived from comparisons with wild type littermates.

[a] CRH levels in patients were measured in the cerebrospinal fluid (after lumbar puncture), in mice in situ hybridization or immunohistochemistry in the PVN. The HPA system in human depression and anxiety was challenged by CRH injection, not by stress. Locomotion as a behavioral parameter was not applicable to humans. Despair in the animals refers to a giving-up strategy in the Porsolt forced swim test. The numbers before the mouse strains refer to the numerical order in the text (Tg = transgenic, n.d. = not done).

primary chronic absence of corticosterone, as well as primary chronic exposure to high corticosteroid levels lead to significant alterations of the serotonergic system [49,50]. Thus, monoamine abnormalities, rather than being a core etiological feature of depression, can also be regarded as secondary to HPA system overdrive. In contrast to depressive illness, the HPA system has been less extensively studied in different types of anxiety disorders. Panic disorder as one of the most 'stress-related' anxiety disorders does not result in baseline changes of the HPA system (Table 1) [12,51]. However, dysregulations of the HPA system have been described in patients with panic disorder following challenge tests, e.g., with ACTH or dex/CRH (Table 1) [12,51]. These changes, however, are relatively mild as compared with the alterations observed in depressive patients.

Human studies of corticosteroid receptor expression and function in the brain have been hampered by the fact that no ligands for PET or SPECT studies are available. Therefore, expression, ligand binding and function (e.g., dexamethasone response) of GRs expressed in human leukocytes were evaluated as markers for GR regulation in the brain. Overall, these studies showed heterogeneous results: some could demonstrate decreased numbers or functional impairments of the GR in depressive patients, while others could not reproduce these changes (for review, see Ref. [52]). In contrast, preliminary results in patients with panic disorder suggested an increased number of lymphocytic GRs [51]. At present, it is not clear, however, as to what extent the expression or functional state of GRs in leukocytes corresponds to that of GRs in the brain. Escape from this dilemma could come from animal models that reflect molecular, biochemical, pharmacological or behavioral features of depression or anxiety.

5. Animal models of depression and anxiety

For many years, animal models of depression were most frequently used by the pharmaceutical industry in screening tests for the development of novel antidepressants. Increasingly, however, such animal models are also being used to investigate the neurobiology of depression. There are some important features an animal model should possess for simulating human depression. The model should employ realistic inducing conditions, model core symptoms of depressive illness and respond to antidepressant drug treatment [15,16]. One of the core symptoms described in DSM-IV, the diagnostic manual of the American Psychiatric Association, is anhedonia. Anhedonia has been proposed to reflect a decrease in the sensitivity of brain reward mechanisms, which can be modelled in animals. Two paradigms have been established in mice that fulfill the criteria of anhedonia: learned helplessness and chronic unpredictable mild stress. In the learned helplessness paradigm, animals are exposed to a series of unpredictable and inescapable electroshocks [53]. As a result, a proportion of animals display impairments of rewarded behavior (sucrose preference), deficits in avoidance learning as well as vegetative symptoms such as decreased appetite and loss of body weight. In the chronic mild stress model, animals are submitted over a prolonged period (several weeks) to a series of mild stressors (intermittent food and water deprivation, overnight illumination, cage tilt, noise, etc.) [54,55]. This treatment induces decreased sucrose consumption, decreased self-stimulation, altered sexual and aggressive behavior, decreased body weight and sleep disturbances, thereby mimicking human depressive symptoms. Both depression models also show an overactivation of the HPA system. Thus, they combine behavioral and neuroendocrine features of human depression.

Due to its fast and uncomplicated applicability, the so-called 'behavioral despair' test has been utilized as a depression paradigm in several strains of genetically manipulated mice. In this model, mice are forced to swim in a restricted space and rapidly adopt a characteristic immobile posture, which is interpreted as a 'depressive' giving-up strategy [56]. However, the behavioral despair test is of relatively low validity as simulation of depression and is mainly used for antidepressant screening [15]. A conceptually similar model, which is also based on an immobility response to inescapable aversive stimulation, is the tail suspension test [57]. As in the behavioral despair test, immobility is reduced by a wide variety of antidepressants. All animal depression models mentioned so far have one thing in common: they are induced by external, i.e., environmental, stimuli, and in this way avoid premature suppositions about the neurochemical correlates of the illness. In contrast, other paradigms used in mice for studying the neurobiology of depression-like pharmacological treatment with reserpine or amphetamine or the olfactory bulbectomy model are based on primary and strong alterations of the animals' physiology. Such models are closely predicated on presupposed neurobiological mechanisms, and are therefore too restrictive to be used in early genetic research [16].

Most behavioral studies in transgenic mice have focused on tests for anxiety. Usually, these tests are designed as approach–avoidance–conflict and measure time and frequency of the animals entering an aversive, anxiety-related maze compartment [17]. Tests like the open field, elevated plus-maze or O-maze, dark–light box test and others are easily to perform and offer robust results [58]. A broad overlap of symptoms between depressive illnesses and anxiety disorders exists in human psychopathology. This is reflected by the fact that some classical antidepressive drugs are also effective in anxiety disorders. It has to be determined, whether in animal models — as one might expect — 'depression' also coincides or correlates with 'anxiety.' On the neuroendocrine level, it has been demonstrated that genes that are dysregulated during depression, like CRH, also play a

major role in the development or maintenance of anxiety disorders [7]. The use of animal models based on genetic manipulations offers both the possibility of identifying the genetic determinants of psychiatric diseases and the making of models that mimic more closely the clinical phenomena [16].

6. Mice with targeted mutations of GR and MR

Introduction of genes into the germ line by transgenic techniques or disruption of genes by homologous recombination (or the combination of both) offers the possibility of generating animal models of human genetic diseases or investigating whether a specific gene participates in the pathogenesis of a disease. In recent years, several mouse strains with specific genomic alterations of the GR and the MR have been generated. These lines can be used to study regulatory mechanisms of the HPA system as well as behavioral consequences of the genetic modification. The following strains have been used for these purposes.

6.1. Transgenic mice with decreased GR expression

In this mouse line, endogenous GR gene expression is decreased by transgenic expression of an antisense RNA directed against the GR under the control of a human neurofilament promoter [60]. The exact mechanism leading to an intracellular reduction of GR mRNA in these mice remains unclear. It has been suggested that the antisense RNA forms hybrids with the endogenous mRNA, resulting in a specific decrease of the targeted mRNA and therefore a decrease of the corresponding protein. When using this approach, it has to be kept in mind that the gene product (GR protein) is not completely abolished. The amount of GR is reduced to an extent that depends on the promoter activity of the antisense transgene and will vary from cell to cell.

6.2. Mice with disrupted alleles of the GR

Two distinct disruptions of the GR gene were generated in mice by gene targeting. The first was achieved by insertion of a neomycin cassette into exon 2 of the GR gene, resulting in a hypomorphic allele [61]. In these mice, an mRNA splice variant persists, which encodes an N-terminal truncated protein containing the DNA-binding domain and the ligand-binding domain [18]. The second mutation results in deletion of a DNA segment that contains exon 3 of the GR gene (GR^{null}; Fig. 3b). This exon encodes the first zinc finger of the DNA-binding domain. Its absence leads to a complete inactivation of the GR gene. Homozygosity of this mutation ($GR^{null/null}$) is incompatible with survival to adulthood. $GR^{null/null}$ mice die a few minutes after birth due to severe atelectasis of the lungs.

6.3. Mice with a nervous-system-specific knockout of the GR (GR^{NesCre})

Since the complete inactivation of the GR caused postnatal lethality, Tronche et al. [62] generated a conditional allele of this gene using the Cre/loxP recombination system in mice. This genetic tool enables the selective disruption of a gene in specific cell types without affecting its activity in other cells of the organism. Cre recombinase is a prokaryotic enzyme that catalyses the excision of DNA fragments flanked by two 34-bp DNA targets, the so-called loxP sites [63]. To generate a cell type or organ-specific gene inactivation, the gene of interest has to be flanked with loxP sites in a way that does not affect normal expression and function. The subsequent transgenic expression of the Cre recombinase under the control of a cell type or organ-specific promoter leads to a selective disruption of the gene in the target tissue, while its function remains intact in the rest of the organism. To obtain a nervous-system-specific inactivation of the GR gene, exon 3 was flanked with loxP sites (GR^{lox}). Mice with this mutation were crossed with mice expressing the Cre recombinase under the control of the rat nestin gene promoter (Fig. 3c). This strategy resulted in viable mice lacking GR in neurons and glial cells (GR^{NesCre}) and allowed the selective study of the role of GR in the nervous system [62].

6.4. Knock-in mice with a DNA-binding defective GR (GR^{dim})

GR controls transcription by two major modes of action: (i) as dimer, binding to positive and negative GREs in the promoter of target genes; (ii) as monomer, modulating the activity of other transcription factors via protein–protein interactions (Fig. 1) [64]. The two modes of action can be dissected by introducing a point mutation (A458T) into the D-loop, i.e., one of the dimerization domains of the GR [65]. Using a knock-in strategy replacing the endogenous GR gene, this mutation was introduced in mice (Fig. 3d) [66]. These so-called GR^{dim} mice express GR molecules that cannot dimerize, but still act as monomers. Consequently, GR^{dim} mice are deficient in activating GRE driven genes, but proficient in the modulation of other transcription factors, e.g., AP-1 and NF-kB [66,67]. In contrast to mice carrying disrupted alleles of GR, GR^{dim} mice are viable and can be used to study physiology and behavior in adulthood [66].

6.5. Transgenic mice with increased GR expression (YGR mice)

GR overexpression in this mouse line was achieved by transgenic expression of two additional copies of the GR gene using a yeast artificial chromosome [68]. Interestingly, the expression level of the GR did not reach the theoretical

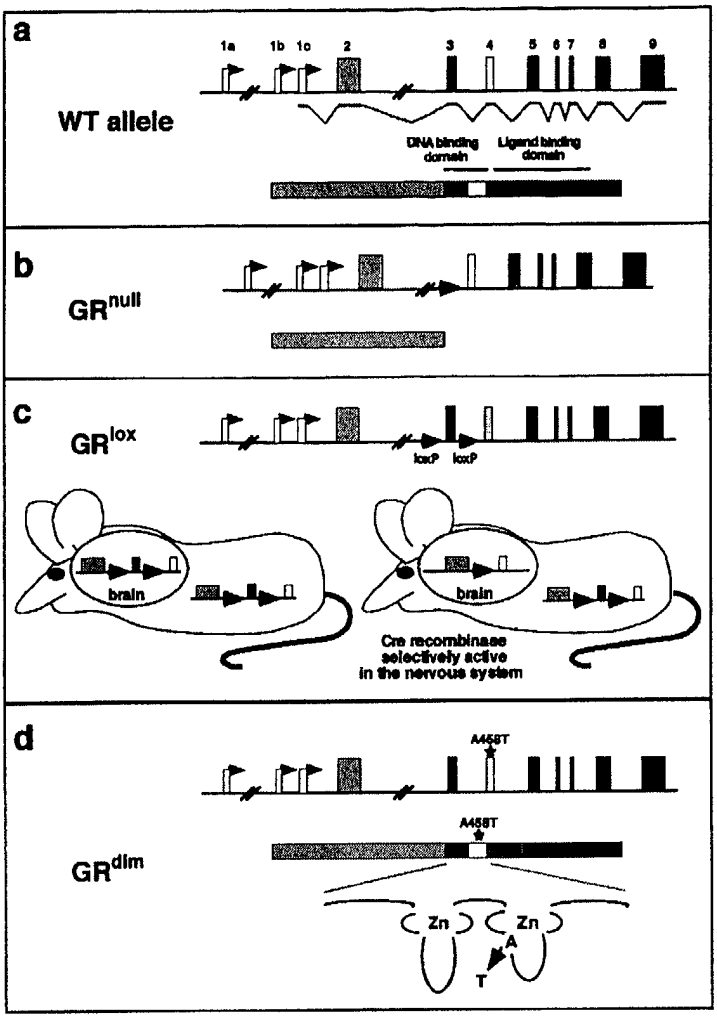

Fig. 3. Schematic representation of different GR alleles used for homologous recombination in mice. (a) Organisation of the wild type (WT) GR gene. The upper scheme depicts the genomic structure of the GR gene with introns and numbered exons and the resulting mRNA. The lower scheme with the bars corresponding in grey to the respective exons represents the translated GR protein. The functional domains resulting from exons 3 and 4 (DNA-binding domain) and exons 5–9 (ligand-binding domain) are indicated. (b) The GR^{null} allele was obtained by deleting the third exon (and a subsequent frame shift), leading to a truncated GR protein without the important functional domains. (c) The GR^{lox} allele was generated by flanking exon 3 with loxP sites. This modification does not primarily impair expression and function of the GR gene but is sensitive to the (artificial) cellular expression of the enzyme Cre recombinase. When the Cre recombinase transgene is expressed in the same cell as GR^{lox}, the latter is turned into GR^{null} by deletion of exon 3 and a subsequent frame shift. When the Cre recombinase is expressed under the control of a promoter specific for the central nervous system, GR is deleted selectively in the brain and spinal cord, but is still normally expressed outside the nervous system. (d) The GR^{dim} allele harbors a point mutation in exon 4 (alanine to threonine) that prevents the dimerization of the GR protein. This strategy selectively eliminates GR functions that require binding to GREs (cf. Fig. 1, modified from Ref. [18]).

twofold elevation predicted. The highest level of overexpression in this mouse strain was observed in brain and pituitary in which GR mRNA was elevated by 60% and 43%, respectively. This demonstrates that GR mRNA expression is subject to autoregulation.

6.6. Knockout mice with disruption of the MR gene

MR −/− mice were generated by classical homologous recombination [59]. When untreated, MR −/− mice develop pseudohypoaldosteronism after birth and die between postnatal days 8 and 13 due to severe renal loss of sodium and water. However, according to a recently established protocol, MR −/− animals can be rescued by exogenous salt supply and studied during adulthood [69,70].

7. HPA system dysregulation and behavioral symptoms in mice with targeted mutations of GR and MR

Alterations in the regulation of the HPA system in mouse strains with genetic manipulations of the GR or the MR as

well as some of their behavioral abnormalities are summarized in Table 1.

7.1. Transgenic mice with decreased GR expression

Initial studies reported an upregulation of the entire HPA system in these animals [60,71]. These results could not be reproduced in subsequent studies, most likely because the first studies were not performed under unstressed conditions. In unstressed, nonstimulated transgenic mice, no differences in plasma ACTH and corticosterone levels were observed when compared to wild type animals, neither at early morning (nadir) nor at evening time (peak) [72–74]. These findings were consistent with unchanged pituitary ACTH and adrenal corticosterone contents in these mice. Thus, under baseline conditions, the HPA system seems not to be altered in these animals. After a CRH challenge, however, transgenic mice with decreased GR expression showed a hyperresponse in ACTH and a blunted response in corticosterone levels, just opposite to that seen in depressive patients [73]. However, transgenic mice are nonresponders in a dexamethasone suppression test, similar as observed in patients with major depression [73]. In contrast to depressive illness, transgenic mice with decreased GR expression exhibit a clear reduction of CRH expression in the hypothalamus [72,74]. Thus, despite some similarities with findings in depressive patients, the transgenic animals show major differences to the neuroendocrine features observed in this psychiatric illness. This interpretation is in line with results in behavioral tests. Transgenic mice with decreased GR expression were clearly less anxious in the elevated plus-maze, where they showed more entries and spent more time in the anxiety-related open compartments [75]. When exposed to intense psychological stress, these mice revealed less anxious behavior such as immobility or staring [76]. In the Porsolt forced swim test, transgenic mice demonstrated significantly *less* floating behavior [75]. Floating is regarded as a 'depressive' giving-up or despair strategy. Furthermore, the animals exhibited cognitive deficits in a short-term olfactory and a long-term spatial memory task [75,77]. The authors concluded that neuroendocrine and behavioral findings appear, to a large part, to be determined by the reduced CRH levels [74]. This interpretation is supported by the anxiolytic effects observed in pharmacological and genetic experiments with transgenic mice in which CRH signalling via CRH receptor-1 was impaired [78–81]. Antidepressant pharmacotherapy reversed all dysfunctions in the behavioral tests in transgenic mice with decreased GR expression. It remains to be determined whether this effect correlates with alterations in CRH and/or GR expression in specific brain regions.

7.2. Mice with disrupted alleles of the GR

Newborn mice homozygous for a GR^{null} allele demonstrate enhanced transcription of both CRH in the hypothalamus and proopiomelanocortin (POMC) in the anterior lobe of the pituitary (unpublished data). Similar results were also obtained in mice homozygous for a hypomorphic GR allele [82]. These results confirm the role of the GR-mediated negative feedback in the HPA system via transcriptional repression. It is not clear from these studies, however, whether feedback control is exerted by DNA-binding-dependent or -independent mechanisms of the GR (cf. Fig. 2). As already pointed out, mice with disrupted GR die postnatally and cannot be subjected to behavioral testing.

7.3. Mice with a nervous-system-specific knockout of the GR (GR^{NesCre})

The selective loss of GR in the nervous system caused a strong activation of the HPA system with markedly elevated levels of circulating corticosteroids (Fig. 4) [62]. The morning basal values of corticosterone were more than 10-fold higher in GR^{NesCre} mutants than in control animals.

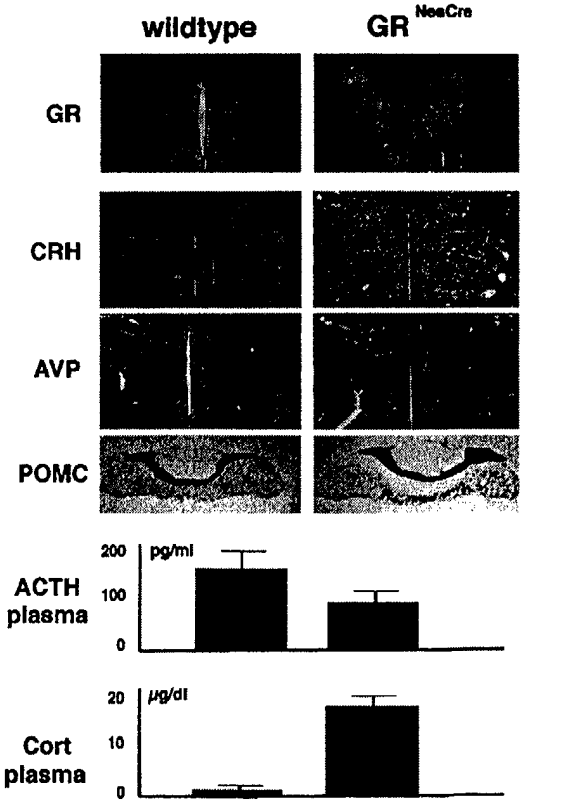

Fig. 4. HPA system activity in GR^{NesCre} mice. As demonstrated by immunocytochemistry, GR is absent in neurons of the hypothalamic PVN (outlined by arrowheads; 3v = 3 ventricle) in GR^{NesCre} mice. This leads to overexpression of CRH protein in the PVN in mutant mice (see high-power inset), but does not affect expression of AVP. Consequently, POMC (encoding ACTH) transcription in the anterior lobe of the pituitary (marked by asterisk) is significantly increased in GR^{NesCre} animals, as demonstrated by in situ hybridization. However, the circulating levels of ACTH are reduced. In contrast, strong elevation of plasma corticosterone is observed in GR^{NesCre} mice (modified from Ref. [62]).

The absence of central negative feedback (despite elevated corticosterone levels) due to the lack of the GR resulted in a strong increase of CRH mRNA and protein in the hypothalamic PVN (Fig. 4). In contrast, expression of vasopressin was not affected. CRH expression outside the hypothalamus, e.g., in the central nucleus of the amygdala, was also not altered in GR^{NesCre} mice. Elevated levels of CRH in PVN and median eminence evoked increased expression of POMC mRNA and protein in the corticotrophs of the anterior pituitary. Since GR expression is preserved in pituitary corticotrophs of GR^{NesCre} mice, GR-mediated repression of POMC in pituicytes is obviously overcome by the elevation of CRH. However, levels of circulating ACTH were significantly reduced in GR^{NesCre} mice. This is most probably due to the intact (GR-mediated) suppression of ACTH secretion. The discordance between decreased circulating levels of ACTH and increased levels of corticosterone may be caused by an increased ACTH sensitivity of the adrenals or a direct stimulation of this gland (possibly by splanchnic innervation or direct CRH effects) that may develop under a chronic hyperactivation of the HPA system. Interestingly, a similar discrepancy in ACTH and cortisol plasma levels has been reported in patients with major depression [83]. Despite the severe hyperactivation of the HPA system, corticosterone levels in GR^{NesCre} mice displayed a preserved but blunted circadian rhythm. Furthermore, the HPA system was still responsive to acute immobilization stress, leading to increased levels of both circulating ACTH and corticosterone. Since elevated corticosterone levels could still act on peripheral tissues expressing GR, GR^{NesCre} mice exhibited features reminiscent of those observed in human Cushing's syndrome.

In behavioral studies, the absence of GR signalling in the brain of GR^{NesCre} mice correlated with a reduction of anxiety. Thus, GR^{NesCre} mice went faster into and spent more time in the aversive bright compartment of the dark–light box (Fig. 5) [62]. Similarly, mutant animals spent significantly more time than controls on the anxiety-related open segments of the elevated O-maze (Fig. 5). In the behavioral despair test, mutant mice demonstrated significantly less floating behavior during the re-test phase, i.e., at the second day of testing. This finding may suggest that GR^{NesCre} mice are less prone to develop 'depressive' behavior over time when repeatedly exposed to external stress. However, since mutant mice also exhibited a mild memory deficit in the Morris water-maze task (unpublished data), the re-test findings in the forced swim test may also reflect cognitive deficits.

7.4. Knock-in mice with a DNA-binding defective GR (GR^{dim})

Using GR^{dim} mice, it was possible to dissect DNA-binding-dependent and -independent mechanisms of the negative feedback control of the GR on the HPA system at its different anatomical levels [66]. The importance of GR monomers in the HPA system feedback control was underlined by twofold increases in corticosterone plasma levels in GR^{dim} mice. However, in sharp contrast to GR^{NesCre} mice, GR^{dim} mice showed normal levels of CRH mRNA and protein in PVN and median eminence, indicating that regulation of CRH expression is independent of GR dimerization, despite a recently identified nGRE in the CRH promoter (Fig. 2) [84]. Despite unchanged levels of CRH, the regulation of the POMC gene was severely altered in GR^{dim} animals. POMC mRNA was strongly upregulated in the anterior pituitary of GR^{dim} mice, and consequently, ACTH immunoreactivity was more than twofold elevated [66]. Expression of prolactin was also

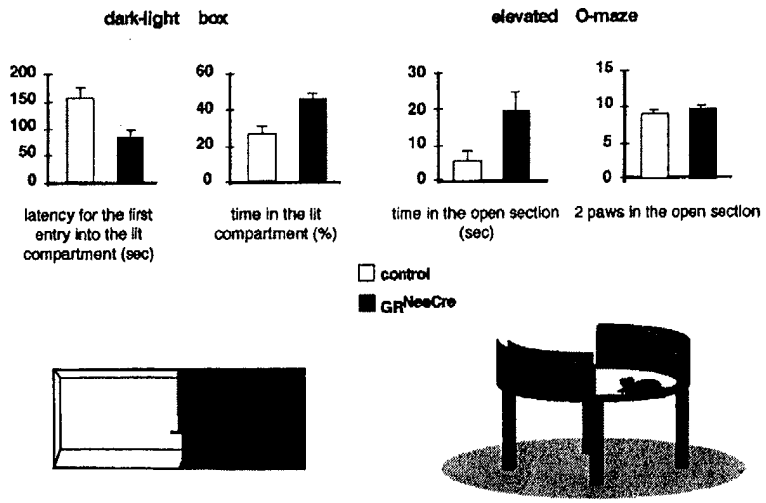

Fig. 5. Reduced anxiety-related behavior in GR^{NesCre} mice. When compared to control, GR^{NesCre} animals show a lower latency to enter and spend more time in the anxiety-related bright compartment during a dark–light test. Similarly, mutant mice spend more time on the aversive open segment of an O-maze (modified from Ref. [62]).

upregulated in the anterior pituitary, suggesting a common mechanism of transcriptional regulation of POMC and prolactin. For both genes, functional nGREs requiring DNA binding of GR have been described [85,86]. Despite increased pituitary ACTH expression, plasma levels of ACTH were unaltered in GR^{dim} mice. Since similar findings were already observed in GR^{NesCre} mice with intact GR function in the pituitary, the release of ACTH (in contrast to its synthesis) seems to be controlled by a dimerization-independent mechanism (Fig. 2). When behaviorally tested, GR^{dim} mice revealed normal locomotion, exploration and anxiety-related behavior. Furthermore, the mutants exhibited the same amount of floating in the behavioral despair test as their wild type littermates. In contrast, mutant animals displayed deficits in spatial memory (Morris water-maze, Oitzl et al., unpublished data). Together with the findings reported for GR^{NesCre} mice, this may suggest that emotional behavior and learning tasks are influenced by GR via different molecular modes of action: learning and memory by GRE- or nGRE-dependent mechanisms, anxiety-related behavior via protein–protein interactions of GRs. An electrophysiological correlate for the learning deficits in GR^{dim} mice may be altered calcium currents or a decreased serotonin responsiveness [87].

7.5. Transgenic mice with increased GR expression (YGR mice)

Overexpression of GR caused a strong suppression of the HPA system with reduced expression of CRH in the hypothalamus, decreased POMC expression in the anterior lobe of the pituitary and diminished corticosterone levels in the plasma [68]. These results reflect an increased GR negative feedback control in the HPA system. As could be expected, YGR mice show the opposite dysregulatory effects of the HPA system as GR^{NesCre} mice (see Table 1). So far, YGR mice have not been studied in behavioral tests.

7.6. Knockout mice with disruption of the MR gene

MR −/− mice show an upregulation of the whole HPA system, with elevated CRH levels in the PVN, higher amounts of POMC and ACTH in the anterior pituitary and significantly elevated plasma corticosterone levels (unpublished observations). Since MR is not expressed in the HPA system itself, higher brain centers, e.g., the hippocampus, are responsible for these effects. In order to study adult MR −/− mice, animals had to be rescued postnatally by exogenous salt supply. This involved daily handling of the animals during the complete early life phase, which may have had profound effects on both HPA system and behavior [88]. However, handling and exogenous salt supply were not crucial factors for the HPA system overactivity in adult MR −/− mice, since this upregulation was already detected in MR −/− embryos at day E18.5. At this timepoint of development, the HPA system feedback regulation is well established, but salt and water homeostasis is maintained via the placental circulation [82]. Embryonic MR −/− animals, however, cannot serve as 'handling controls' for behavioral testing. Initial experiments suggested increased anxious behavior in salt rescued adult MR −/− mice. Due to the severe caveats mentioned, a thorough behavioral analysis needs to be performed in animals with a more elaborate (i.e., a brain- or hippocampus-specific) genetic disruption of the MR gene.

8. Mice with targeted mutations of GR and MR: models for anxiety and depression?

Mice with targeted mutations of the GR are candidate models for anxiety disorders. One argument is the fact that all transgenic strains discussed in this review — with the exception of GR^{dim} mice — demonstrate alterations in anxiety-related behavior. Decreased or absent GR levels in the brain coincide with reduced anxiety-related behavior. In the transgenic mice with decreased GR expression, the reduced anxiety could be simply caused by the decreased levels of CRH because this hormone has well-documented anxiogenic effects. Thus, centrally delivered as well as genetically overexpressed CRH leads to enhanced anxiety, while inactivation of the CRH receptor 1 gene in mice causes a reduction of anxiety [80,81,89]. In GR^{NesCre} mice, however, CRH is upregulated in the PVN neurons and unchanged in the central nucleus of the amygdala. Therefore, the reduction of anxiety-related behavior in these mutants — despite potential anxiogenic effects of upregulated CRH — suggests a direct participation of glucocorticoids and their receptors in anxiety modulation. This concept provokes the hypothesis that MR mutant mice could also be more anxious due to a combination of upregulated CRH and increased GR signaling via elevated corticosterone plasma levels. The molecular mechanisms by which the GR influences anxiogenic behavior remain to be identified. For this purpose, GR^{NesCre} mice represent a valuable tool, since they can be used for studying the (altered) expression of candidate molecules.

The usefulness of mice with targeted mutations of the GR as models for depression is currently less clear because most of these mice have not been tested for the symptoms that most closely reflect human depression (see above). As 'homologous' (i.e., genetic) models for depression, they should be studied for the presence of the core symptoms, anhedonia and despair. Testing these strains in a predictive (i.e., stress-induced) model of depression should elaborate whether their genetic defect renders them more prone or resistant to develop behavioral symptoms of depression. The effects of antidepressive pharmacotherapy have so far only been studied in transgenic mice with decreased GR expression [75]. In these mice, treatment with a reversible inhibitor of monoamine oxidase A (moclobemide) reversed their

behavioral deficits. However, these deficits were decreased anxiety and reduced despair, which means that the pharmacological treatment was therapeutic but by no means 'antidepressive.' GR^{NesCre} mice seem to represent a valuable tool to dissect the effects of increased CRH and its receptors from those of GR and elevated corticosterone. This could clarify the important question of whether a key biological feature of major depression in humans — increased hypothalamic and cerebrospinal CRH — is a primary or a secondary feature [8]. Despite an increased CRH expression, GR^{NesCre} mice demonstrate reduced anxiety-related behavior. A first hint that they may also exhibit antidepressive features comes from the behavioral despair test where they reveal less 'depressive' floating than their wild type littermates. However, GR^{NesCre} mice have to be subjected to behavioral tests more closely imitating the features of human depression such as learned helplessness or chronic unpredictable mild stress. Most concepts of corticosteroid receptor involvement in psychiatric disease states have focused on the GR. However, it has been also hypothesized that reduced capacity of the hippocampal MR is involved in the HPA system dysregulation found in depression and aging. Healthy test persons treated with the MR antagonist spironolactone showed increased HPA system activity and a dysregulation of the dex/CRH test reminiscent of changes observed in human patients [90]. Spironolactone, when coadministered to antidepressant pharmacotherapy, impairs the patients' drug response [7]. First studies in MR−/− mice confirm a hyperactivation of the HPA system with both increased CRH expression and elevated GR-mediated signaling via raised corticosterone levels. Again, behavioral experiments in mice with compromised MR expression or function are necessary to promote or reject a role of this receptor in the etiology or pathogenesis of depression.

9. Potential molecular consequences of corticosteroid receptor deficiency

Of the many postulated and conceivable molecular downstream effects of GR-mediated signaling, only two potentially related effects will be discussed in this review. Recent studies have demonstrated that stress can decrease the expression of brain-derived neurotrophic growth factor (BDNF) in hippocampal granule cells [91,92]. Furthermore, a decreased volume of the hippocampus — most likely due to dendritic atrophy of vulnerable CA3 hippocampal neurons — has been documented in stress-exposed animals as well as in patients with major depression [93,94]. In contrast, infusions of BDNF into the adult rat brain produce sprouting of (serotonergic) nerve terminals [95]. Furthermore, treatment with antidepressant drugs has the opposite effect as stress in hippocampal granule neurons: an upregulation of BDNF expression [96]. This upregulation, in turn, should lead to a trophic response of CA3 neurons, which are the targets of dentate gyrus granule cells. Thus, the hypothesis has been put forward that BDNF could be a molecule whose up-or downregulation mediates antidepressive or depressive effects, respectively [97]. Behavioral studies using mice with genetically reduced BDNF levels, however, showed regular spatial learning and inconspicuous anxiety-related behavior (elevated T-maze), but the animals have not been subjected to a despair or depression paradigm [98]. For a closer analysis of BDNF and its regulation in stress-related disorders, transgenic animals with compromised corticosteroid receptor functions represent a valuable tool to prove a link between this potential target gene and GR-mediated behavioral alterations. If the aforementioned hypothesis holds true, one would expect a strong correlation between the development of depressive behavior and BDNF downregulation, or alternatively, between stress-resistant behavior and BDNF upregulation, in mice mutant for GR or MR. Recent studies in rats have given first indications for such a correlation. Hippocampal BDNF expression shows a diurnal regulation that mimics with a clear phase shift the circadian rhythm of endogenous glucocorticoids [99]. Furthermore, chronic infusion of high doses of BDNF improved the outcome in the learned helplessness paradigm [100].

Another important molecular linkage in the neurobiology of stress and depression are the reciprocal influences of the glucocorticoid and the monoaminergic systems [101]. Thus, acute or chronic stress up- or downregulates various components of the serotonergic system, such as tryptophan hydroxylase, serotonin transporter or various serotonin receptor subtypes [102–105]. On the other hand, antidepressant drugs modulate the expression levels of GR, MR and their ratio [106–108]. Functional studies have shown that the balance between activation of MR and GR determines the response of the hippocampus to activation of the serotonergic raphe–hippocampal pathway. The hypothesis has been put forward that the balance between MR and GR activation is altered during chronic stress or depression, resulting in a condition of combined hypercorticism and an apparent hypoactivity of serotonergic neurotransmission (for review, see Refs. [25,49]). Similar as it has been pointed out for BDNF expression studies, mice with targeted mutations of corticosteroid receptor genes are appropriate models that could establish a causal relationship between alterations of MR- or GR-mediated signaling, changes of serotonergic neurotransmission and potential behavioral abnormalities.

10. Conclusions

Targeting of corticosteroid receptor genes by homologous recombination in embryonic stem cells and by transgenic approaches has generated several strains of mutant mice with lost or altered function of GR and MR. These mice allow the in vivo study of causal effects of the disrupted genes, and thus enable a correlation between GR or MR functioning and expression of target genes, endocrinology and behavior. Such endocrinological studies have

yielded valuable insights into different feedback mechanisms of the HPA system controlled by MR and GR, respectively. The generation of dimerization-defective GR^{dim} mice allowed to distinguish between transcriptional actions of GR dimers at the level of the pituitary and effects as monomer at the level of the hypothalamus (Fig. 2). In contrast, the MR exerts its control over the HPA system selectively at higher brain centers such as the hippocampus.

Since dysfunction of the central stress hormone system is causally involved in the pathogenesis of depression and anxiety, mice with disrupted GR or MR can serve as models, which mimic symptoms of these psychiatric disorders. Behavioral analyses of GR mutant mice revealed that emotional responses are controlled by GR monomers, since GR^{NesCre}, but not GR^{dim}, mice exhibited a significant decrease of anxious behavior in several tests. Therefore, mice with targeted mutations of the GR are candidate models for anxiety disorders. So far, however, none of the mutants described here can be viewed as an animal model of a specific psychiatric disease defined by common set of diagnostic criteria. Such criteria can be and have to be developed for mice. Genetic models for depression should demonstrate the presence of the core symptoms, anhedonia and despair. Further testing in a stress-induced model of depression should elaborate whether a specific mutation renders the GR or MR mutant mice more prone or more resistant to develop behavioral symptoms of depression. Such studies will be performed in the mouse strains reviewed here in oncoming months.

Using promoters with neuroanatomically more restricted activity than the nestin promoter will lead to the identification of brain regions where GR and MR are involved in the symptomatology of affective disorders. Furthermore, molecular neurobiology will soon allow an inducible mutagenesis in adult mice. This will enable experiments in which the behavior of an individual mouse can be studied before and after gene disruption. This progress may overcome the caveats against conventional gene-targeting techniques concerning the influence of the genetic background of breeding animals and stem cells, the developmental compensation of mutations, etc. [25,109,110]. DNA microarray technology will be used for the identification of target genes regulated by GR and MR in brain areas responsible for specific symptoms of stress-related disorders. Such corticosteroid receptor-regulated genes may code for proteins that could turn out to represent new drug targets for the treatment of depression and anxiety.

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